(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number : 0 461 863 A1

(12) **EUROPEAN PATENT APPLICATION**

(21) Application number : 91305270.0

(22) Date of filing : 11.06.91

(51) Int. Cl.[5] : C07H 21/04, C12Q 1/70, C12Q 1/68, C12N 15/51, C12P 19/34

(30) Priority : 12.06.90 JP 153402/90

(43) Date of publication of application : 18.12.91 Bulletin 91/51

(84) Designated Contracting States : BE CH DE DK FR GB IT LI NL

(71) Applicant : IMMUNO JAPAN INC.
4-28-14-701, Ogikubo Suginami-Ku
Tokyo 167 (JP)

(72) Inventor : Okamoto, Hiroaki
3132-24, Yakushi-ji, Minami Kawachi Machi
Kawachi-Gun, Tochigi-ken (JP)
Inventor : Nakamura, Yatsuo
4-28-14-701, Ogikubo, Suginami-ku
Tokyo 167 (JP)

(74) Representative : Arthur, Bryan Edward et al
Withers & Rogers 4 Dyer's Buildings Holborn
London EC1N 2JT (GB)

(54) **Oligonucleotide primers, and their application for high-fidelity detection of non-A, non-B hepatitis virus.**

(57) The noncoding region, containing 324 nucleotides, of the 5' terminus of non-A, non-B hepatitis virus is disclosed. Also disclosed is the nucleotide sequence of the structural gene which is upstream of the noncoding region. Oligonucleotide primers derived from these regions can be used to detect non-A, non-B hepatitis virus.

EP 0 461 863 A1

Introduction to the Invention

The present invention concerns high-fidelity detection of non-A, non-B hepatitis virus (hereinafter called NANB hepatitis virus) and oligonucleotide primers used in a detection system for detecting NANB hepatitis virus.

Viral hepatitis of which DNA and RNA have been elucidated include hepatitis A, hepatitis B, hepatitis D and hepatitis E. However, in spite of great efforts by scientists the world over, the causative virus of NANB hepatitis (which is mainly caused by blood bourne infection) falls in none of the above groups and has not been isolated.

In 1988, Chiron Corp. reported that it had succeeded in cloning the RNA virus genome of the causative agent of NANB hepatitis (which it termed hepatitis C virus (hereinafter called HCV)) and disclosed part of the nucleotide sequence of HCV. HCV antibody detection systems based on that sequence are now being introduced for screening of blood for transfusion and for diagnosis of patients.

However, the nucleotide sequence disclosed by Chiron Corp. was only part of the NANB hepatitis viral genome. Moreover, it was part of a sequence of relatively little importance. HCV antibody detection systems developed on the basis of that sequence, therefore, fail to provide both sufficient sensitivity and specificity for NANB hepatitis virus and for therapy and prognosis of acute and chronic NANB hepatitis, although such systems have proven their partial association with NANB hepatitis.

More than 95% of posttransfusion hepatitis cases in Japan are NANB hepatitis. There are 280,000 annual estimated cases of this disease. The course of NANB hepatitis is troublesome, with most patients becoming carriers who develop chronic hepatitis. In addition, those patients with chronic hepatitis develop liver cirrhosis and then hepatocellular carcinoma at a fairly high rate over 10 to 20 years. Therefore it is imperative to isolate the virus itself and to develop effective diagnostic reagents enabling earlier diagnosis.

As described earlier, there are significant numbers of patients with acute or chronic NANB hepatitis which can not be diagnosed by the detection systems using Chiron's HCV antibody. For accurate diagnosis of these cases of hepatitis, detection systems for the virus based on elucidation of the viral agent at its gene level is required.

Summary of the Invention

An object of the present invention is to provide a highly sensitive detection system for NANB hepatitis virus at its gene level and oligonucleotide primers used for such system.

Brief Description of the Drawing

Figure 1 shows determination method of nucleotide sequences of NANB hepatitis viral RNA.

Detailed Description of the Invention

For the purpose of elucidation of the NANB hepatitis viral gene, the inventors isolated NANB hepatitis viral RNA from human and chimpanzee carrier sera and determined the nucleotide sequence of the 5′ terminus by cloning its cDNA. As a result, the inventors identified that for two different strains the RNA genome had a 5′ noncoding region having a sequence of at least 324 nucleotides. This sequence had not been disclosed by Chiron and is totally novel. It was also determined that the nucleotide sequence in this region was highly conserved among different strains. For example, the RNA of strains HC-J1 and HC-J4 (used for the determination of the nucleotide sequence) differed from each other by only three nucleotides.

On the other hand, large differences (or mutations) in nucleotides were identified in other regions of NANB hepatitis viral RNA. When this fact is taken into consideration, it is amazing that the nucleotide sequence is conserved so well in the 5′ noncoding region. It was further determined that there were few differences in the nucleotide sequence in the upstream part of the structural gene following the noncoding region. Based on these findings, the inventors discovered that use of oligonucleotide primers derived from these regions would detect, with high sensitivity, NANB hepatitis RNA irrespective of the strain.

The present invention, therefore, concerns a NANB hepatitis virus detection system using oligonucleotide primers having nucleotide sequences corresponding to part of the 5′ noncoding region of the viral RNA and/or part of the 5′ side of the region coding for the structural protein of the virus.

The primers may contain from about 15 to about 25 nucleotides, preferably 20.

Abbreviations used in this invention are as follows: for RNA, A, G, C and U stand for adenine, guanine, cytosine and uracil respectively; for DNA, A, G, and C indicate the same bases as in RNA and T stands for

thymine; for polypeptides A, R, N, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y and V are respectively the amino acids of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine.

The inventors have identified that the 5' noncoding region has the following nucleotide sequence (HC-J1 strain):

```
G C C G A C A C T C   C A C C A T A G A T
C A C T C C C C T G   T G A G G A A C T A
C T G T C T T C A C   G C A G A A A G C C
T C T A G C C A T G   G C G T T A G T A T
G A G T G T C G T G   C A G C C T C C A G 100
G A C C C C C C C T   C C C G G G A G A G
C C A T A G T G G T   C T G C G G A A C C
G G T G A G T A C A   C C G G A A T T G C
C A G G A C G A C C   G G G T C C T T T C
T T G G A T A A A C   C C G C T C A A T G 200
C C T G G A G A T T   T G G G C G C G C C
C C C G C A A G A C   T G C T A G C C G A
G T A G T G T T G G   G T C G C G A A A G
G C C T T G T G G T   A C T G C C T G A T
A G G G T G C T T G   C G A G T G C C C C 300
G G G A G G T C T C   G T A G A C C G T G
C A C C
```

The inventors have further determined that the remaining sequence of the 5' noncoding region of the HC-J4 strain is identical to that of HC-J1 (except for the 187th nucleotide A which is replaced by C, the 217th nucleotide C which is replaced by T, and the 226th nucleotide A which is replaced by G). The inventors subsequently developed the highly sensitive detection system for NANB hepatitis virus using oligonucleotide primers corresponding to part of the 5' noncoding region of NANB hepatitis virus. Therefore, any oligonucleotide primer belonging to this defined region of HCV genome may be included in this invention, though replacement of one or more nucleotides is also included.

The inventors have also identified that the upstream region coding for the structural protein of the virus following the aforementioned 5' noncoding region was well conserved among the strains. There were only a few differences between the strains. Nucleotide sequences corresponding to part of that region, used as primers, can detect NANB hepatitis virus with high sensitivity.

The upstream region of the structural gene of the NANB hepatitis virus has the following nucleotide sequence (for the HC-J1 strain):

```
      ATGAGC ACGATTCCCA
AACCTCAAAG AAAAACCAAA
CGTAACACCA ACCGTCGCCC
ACAGGACGTC AAGTTCCCGG400
GTGGCGGTCA GATCGTTGGT
GGAGTTTACT TGTTGCCGCG
CAGGGGCCCT AGATTGGGTG
TGCGCGCGAC GAGGAAGACT
TCCGAGCGGT CGCAACCTCG500
AGGTAGACGT CAGCCTATCC
CCAAGGTGCG TCGGCCCGAG
GGCAGGACCT GGGCTCAGCC
CGGGTACCCT TGGCCCCTCT
ATGGCAATGA GGGCTGCGGG600
TGGGCGGGAT GGCTCCTGTC
TCCCCGTGGC TCTCGGCCTA

GTTGCGGCCC CACGGACCCC
CGGCCTAGGT CGCGCAATTT
GGGTAAGGTC ATCGATACCC700
TCACGTGCGG CTTCGCCGAC
CTCATGGGGT ACATACCGCT
CGTCGGCGCC CCTCTTGGAG
GCGCTGCCAG GGCCCTGGCG
CATGGCGTCC GGGTTCTGGA800
AGACGGCGTG AACTATGCAA
CAGGGAACCT TCCTGGTTGC
TCTTTCTCTA TCTTCCTTCT
```

Except for the following differences, the upstream region of strain HC-J4 has the same nucleotide sequence as HC-J1 (numbers in parenthesis show respective differences in the sequence from the 5' noncoding region):

A (335), T (339), C (375), C (402),
T (405), C (430), C (450), G (453),
T (471), T (501), A (504), T (505),
G (507), A (510), A (513), C (527),
T (528), C (531), A (534), G (547),
T (596), G (597), A (606), A (621),
C (627), C (633), T (675), C (678),
T (701), A (705), T (720), T (732),
T (734), C (753), A (756), G (759),
T (775), A (780), C (783), T (786),
G (801), T (829), G (831), C (834),
C (858), T (859).

As far as oligonucleotide corresponding to part of the upstream region of the structural gene of the virus is concerned, also included are nucleotide sequences with a small number of nucleotides different from strains HC-J1 and HC-J4.

This invention includes detection of NANB hepatitis virus by amplification of cDNA of the viral RNA by Polymerase Chain Reaction (hereinafter called PCR) using oligonucleotide primers disclosed herein. PCR is a method well known in this art.

Under optimum conditions, using the product of the first amplification of cDNA by PCR as a template, a second amplification by PCR is carried out. In the second PCR amplification, a pair of primers that can be annealed inside the first pair of primers is used.

The present invention includes oligonucleotide primers used in the above described detection system. This invention also includes creation of oligonucleotide primers having nucleotide sequences corresponding to part of the 5' noncoding region of NANB hepatitis viral RNA genome (having at least 324 nucleotides), and creation of oligonucleotide primers having nucleotide sequences specific to part of the 5' region coding for the structural protein of the virus.

The following primers are particularly preferable (numbers in parenthesis show positions in sequence from the 5' noncoding region):

#23: T A G A T T G G G T G T G C G C G C G A (450-469 of strain HC-J1),
#25: T C C C T G T T G C A T A G T T C A C G (807-826),
#32: A C T C C A C C A T A G A T C A C T C C (7- 26),
#33: T T C A C G C A G A A A G C G T C T A G (46- 65),
#36: A A C A C T A C T C G G C T A G C A G T (229-248), and
#48: G T T G A T C C A A G A A A G G A C C C (171-190).

When the above primers are in use in PCR, combined use (e.g., of #23 and #25, #32 and #36, or #33 and #48) can enhance the effect of PCR.

This invention also covers NANB hepatitis virus detection systems (e.g., PCR) using the above oligonucleotide primers.

Examples of application of this invention are shown below. However, this invention shall in no way be limited to those examples.

Examples

Example 1 - Determination of the nucleotide sequence of the 5' terminus of NANB hepatitis virus:

(1) Isolation of RNA

RNA was isolated by the method described below from a plasma sample (HC-J1) of a Japanese blood donor who tested positive for HCV antibody and a sample (HC-J4) from a chimpanzee challenged with NANB hepatitis for infectivity but which tested negative for HCV antibody by Ortho HCV Ab ELISA Test (Ortho Diagnostic Systems, Tokyo, Japan).

1.8 ml of each of the plasma samples was added with 1 ml of Tris chloride buffer (10mM, pH 8.0) and centrifuged at 68 x 10^3 rpm for 1 hour. The precipitate was suspended in Tris chloride buffer (50 mM, pH 8.0) con-

taining 200 mM NaCl, 10 mM EDTA, 2% (w/v) sodium dodecyl sulfate (SDS) and proteinase K (1 mg/ml), incubated at 60°C for 1 hour, then extracted by phenol/chloroform and precipitated by ethanol to obtain RNAs.

(2) cDNA synthesis

RNA isolated from HC-J1 plasma was incubated at 70°C for 1 minute and used a template. 10 units of reverse transcriptase (cDNA Synthesis Plus, Amersham Japan) and 20 pmol of oligonucleotide primer (20 mer) were added and incubated at 42°C for 1.5 hours to obtain cDNA. Primer #8 (5'- G A T G C T T G C G G A A G C A A T C A - 3') was prepared by referring to the base sequence shown in figure 47-1 (sequence position 401 to 420) of the European Patent Application No. 88310922.5 (the entire application (now European Patent No. 0,318,216) is incorporated by reference).

(3) cDNA was amplified by the following Polymerase Chain Reaction (PCR).

cDNA was amplified for 35 cycles according to Saiki's method (Science 239, 487-491 (1988)), incorporated by reference in its entirety, using Gene Amp DNA Amplifier Reagent (Perkin-Elmer Cetus) on a DNA Thermal Cycler (Perkin-Elmer Cetus).

(4) Determination of nucleotide sequence by assembling cDNA clones.

As shown in Figure 1, the nucleotide sequence of the 5' termini of the genomes of strains HC-J1 and HC-J4 were determined by combined analysis of clones obtained from the cDNA library constructed in bacteriophage lambda gt10 and clones obtained by amplification of HCV specific cDNA by PCR. Figure 1 shows the 5' terminal sequence of NANB hepatitis virus genome together with cleavage sites of restriction endonucleases and sequences of primers used. In Figure 1, solid lines are nucleotide sequences determined by clones from bacteriophage lambda gt10 library while dotted lines show sequences determined by clones obtained by PCR.

The 1656 nucleotide sequence of HC-J1, spanning nt437-2092, was determined by the clone ø41 obtained by inserting the cDNA synthesized with the primer #8 into bacteriophage lambda gt10 (Amersham).

Primer #25 (5' - T C C C T G T T G C A T A G T T C A C G - 3') of nt 807 - 826 was synthesized based on that ø41 sequence, and 4 clones (ø60, ø61, ø66 and ø75) were obtained to cover the upstream sequence nt1 - 826.

The upstream sequence of strain HC-J1 was determined by clones obtained by PCR using primers #44 (5' - G G C G A C A C T C C A C C A T A G A T - 3') and #25 (5' - T C C C T G T T G C A T A G T T C A C G - 3').

The downstream sequence of 1163 nucleotides, from nt721 up to 1883, of strain HC-J4 was determined by 3 clones (C2821, C3173 and C3192) by PCR using primers #30 (5' - C T C A T G G G G T A C A T T C C G C T - 3') and #42 (5' - T C G G T C G T C C C C A C C A C A A C - 3').

From the analysis described above, nucleotide sequences of the 5' termini of the genomes of strains HC-J1 and HC-J4 were determined as shown below.

The nucleotide sequence of the genome of strain HC-J1 is shown in line (a) and that of strain HC-J4 in line (b), the latter showing only differing nucleotides vis a vis (a). Noncoding region nt1 - 324 is shown in small letters. nt325 - 1863 is a region coding forvarious protein starting with initiation codon ATG and is shown in capital letters (the nucleotide sequence in the aforementioned European Patent Application started only with the 1873th nucleotide, and missed the upstream sequence which is originally revealed in this invention):

```
(a) g g c g a c a c t c   c a c c a t a g a t
(b) - - - - - - - - - -   - - - - - - - - - -

    c a c t c c c c t g   t g a g g a a c t a
    - - - - - - - - - -   - - - - - - - - - -

    c t g t c t t c a c   g c a g a a a g c g
    - - - - - - - - - -   - - - - - - - - - -

    t c t a g c c a t g   g c g t t a g t a t
    - - - - - - - - - -   - - - - - - - - - -

    g a g t g t c g t g   c a g c c t c c a g 100
    - - - - - - - - - -   - - - - - - - - - -

    g a c c c c c c c t   c c c g g g a g a g
    - - - - - - - - - -   - - - - - - - - - -

    c c a t a g t g g t   c t g c g g a a c c
    - - - - - - - - - -   - - - - - - - - - -

    g g t g a g t a c a   c c g g a a t t g c
    - - - - - - - - - -   - - - - - - - - - -

    c a g g a c g a c c   g g g t c c t t t c
    - - - - - - - - - -   - - - - - - - - - -

    t t g g a t a a a c   c c g c t c a a t g 200
    - - - - - - c - - -   - - - - - - - - - -

    c c t g g a g a t t   t g g g c g c g c c
    - - - - - - - - - -   - - - - - - t - - -
```

Mg 1

complementary to Seq ID NO. 4 bp 1-17

Mg 3

Seq ID NO. 4

```
cccgcaagac  tgctagccga
-----g----  ----------
gtagtgttgg  gtcgcgaaag
----------  ----------
gccttgtggt  actgcctgat
----------  ----------
agggtgcttg  cgagtgcccc300
----------  ---------
gggaggtctc  gtagaccgtg
----------  ----------
caccATGAGC  ACGATTCCCA
----------  ----A---T-
AACCTCAAAG  AAAAACCAAA
----------  ----------
CGTAACACCA  ACCGTCGCCC
----------  ----C-----
ACAGGACGTC  AAGTTCCCGG400
----------  ----------
GTCGCCGTCA  GATCGTTGGT
-C--T-----  ----------
GGACTTTACT  TGTTGCCGCG
---------C  ----------
CAGCGGCCCT  AGATTGGGTG
---------C  --C-------
TGCGCGCGAC  GAGGAAGACT
----------  T---------
TCCGAGCGGT  CGCAACCTCG500
----------  ----------
AGGTAGACGT  CAGCCTATCC
T--AT-G--A  --A-------
```

Complementary to seq 2

```
CCAAGGTGCG TCGGCCCGAG
------CT-- C--A------
GGCAGGACCT GGGCTCAGCC
------G--- ----------
CGGGTACCCT TGGCCCCTCT
---------- ----------
ATGGCAATGA GGGCTGCGGG600
---------- -----TG---
TGGGCGGCAT GGCTCCTGTC
-----A---- ----------
TCGCCGTGGC TCTCGGCCTA
A-----C--- --C-------
GTTGGGGCCC CACGGACCCC
---------- ----------
CGGCGTAGGT CGCGCAATTT
---------- ----T--C--
GGGTAAGGTC ATCGATACCC700
---------- ----------
TCACGTGCGG CTTCGCCGAC
-T--A----- ---------T
CTCATGGGGT ACATACCGCT
---------- -T--T-----
CGTCGGCGCC CCTCTTGGAG
---------- --C--A--G-
GCGCTGCCAG GGCCCTGGCG
---------- ----T----A
CATCGCGTCC GGGTTCTGGA800
--C--T---- ----------
AGACGGÇGTG AACTATGCAA
G--------- ----------
```

```
C A G G G A A C C T   T C C T G G T T G C
- - - - - - - - T -   G - - C - - - - - -
T C T T T C T C T A   T C T T C C T T C T
- - - - - - - - - -   - - - - - - - C T -
G G C C C T G C T C   T C T T G C C T G A
- - - T T - - - - G   - - C - - T T - - -
C T G T G C C C G C   T T C A G C C T A C 900
- C A - C - - A - -   - - - C - - T - - T
C A A G T G C G C A   A C T C C A C A G G
G - - - - - - - - -   - - G T G T - C - -
G C T T T A T C A T   G T C A C C A A T G
- A - A - - C - - -   - - - - - G - - C -
A T T G C C C T A A   C T C G A G T A T T
- C - - - T - C - -   - - - A - - C - - -
G T G T A C G A G G   C G C A T C G A T G C
- - - - - T - - - -   - A G C G - - C A T
C A T C C T G C A T   A C T C C G G C G T 1000
G - - - A - - - - -   - - - - - C - - - -
G T G T C G C T T G   C G T T C G C G A G
- C - - G - - C - -   - - - - - - G - - -
G G C A A C G T C T   C G A G G T G T T G
- A - - - - A G - -   - C C - T - - C - -
G G T G G C G A T G   A C C C C C A C G G
- - - A - - - C - C   - - T - - - - - - C
T A G C C A C C A C   G G A C G G C A A A
- C - - G G - - - -   - A - T - C - - G C
C T C C C C G C G A   C G C A G C T T C G 1100
G - - - - - A - T -   - - A C A A - A - -
A C G T C A C A T C   G A T C T G C T T G
- - - C - - - G - -   - - C T - - - - C -
```

```
TCGGGAGCGC  CACCCTCTGT
-T---GCG--  TG-TT----C
TCCCCCCTCT  ACGTGGGGGA
--C--TA-G-  ----------
TCTGTGCGGG  TCCGTCTTCC
---C-----A  --T--T----
TTATTGGTCA  ACTGTTTACC 1200
-CG-CTCC--  G-----C---
TTCTCTCCCA  GGCGCCACTG
-----G--TC  -C--G--TGA
GACAACGCAA  GGCTGCAATT
----GT---G  -A------C-
GTTCTATCTA  CCCCGGCCAT
-C--A-----  T---------
ATAACGGGTC  ATCGCATGGC
T--T-A----  -C--------
ATGGGATATG  ATGATCAACT 1300
T---------  ----------
GGTCCCCTAC  GGCGCCGTTG
----A-----  AA-A--CC-A
GTAATGGCTC  AGCTGCTCCG
--GG--T-G-  --T-------
GATCCCACAA  GCCATCTTGG
----------  --TG--G---
ATATGATCGC  TGGTGCTCAC
-C---G-G--  G--G--C---
TGGGGAGTCC  TGGCGGGCAT 1400
----------  --------C-
AGCGTATTTC  TCCATGCTGG
T--C--C-AT  --------A-
```

```
G G A A C T G G G C   G A A C G T C C T G
- - - - - - - - - -   T - - - - - - - - -
G T A G T G C T G T   T G C T G T T T G C
A - T - - - G C - C   - A - - C - - C - -
C G G C G T C G A C   G C G G A A A C C A
- - - - - - T - - -   - G - - - G - - - T
T C G T C T C C G G   G G G A C A A G C C 1500
A - A C G - - G - -   - - - G G C G - - -
G C C C C C G C C A   T G T C T G G A C T
A G - - A - A - - -   C C - - C A C G - -
T G T T A G T C T C   T T C A C A C C A G
C - C G T C C - - -   - - - T - - - - T -
G C G C T A A G C A   G A A C A T C C A G
- G - - G T C T - -   - - G A - - - - - -
C T G A T C A A C A   C C A A C G G C A G
- - T G - G - - T -   - - - - - - - - - -
T T G G C A C A T C   A A T A G C A C G G 1600
C - - - - - - - - -   - - C - - G - - T -
C C T T G A A C T G   C A A T G A A A G C
- - C - A - - - - -   - - - - - - C T C -
C T T A A C A C C G   G C T G G T T A G C
- - C C - - - - T -   - G - T C C - T - -
A G G G C T T A T C   T A T C A A C A C A
C - C - - - G T - -   - - C A C - - - - -
A A T T C A A C T C   T T C G G G C T G T
G G - - - - - - - -   G - - C - - G - - C
C C C G A G A G G T   T G G C C A G C T G 1700
- - G - - - C - C A   - - - - - - - - - -
C C G A C G C C T T   A C C G A T T T T G
- - - C - C - A - -   G A - T G G - - C -
```

```
ACCAGGGCTG GGGCCCTATC
C------A-- ------C---
AGTCATGCCA ACGGAAGCGG
-CCT--A-TG -GCCTGA-A-
CCCCGACCAA CGCCCCTATT
---C--T--G A-G--T----
GTTGGCACTA CCCCCCAAAA1800
-C-----T-- -G-G--TCG-
CCTTGCGGTA TCGTGCCCGC
--G--T---- ----A-----
AAAGACCGTA TGTGGCCCGG
GTC-CAG--G -----T--A-
TATATTGCTT CACTCCCAGC
-G-------- ---C--A---
CCC1863
--T
```

For the gene region (nt325 - 1863) of strains HC-J1 and HC-J4, the sequences of 513 amino acids encoded were determined and homology, amino acids components and hydrophilicity of the amino acids between the two strains were studied. As a result, region nt325-864 was considered to be coding for the NANB hepatitis virus core proteins. Mutations or differences in the nucleotide sequence in this region were relatively smaller than in other coding regions and approximately 80% of the nucleotide mutations or differences identified were not accompanied by a change in amino acid sequence. Together with the nucleotide sequence of the 5' non-coding region already described above, the sequences of the structural gene region were also helpful in choosing appropriate nucleotide sequences of oligonucleotide primers used for the detection system in this invention. The envelope proteins is considered to be encoded by nucleotides 865-1476 and nonstructural proteins encoded by nucleotides 1477 and above.

The inventors have also identified that among oligonucleotide primers for the core region, the primer #25 (nt807 - 826), which has the least mutations or differences, is the best for the detection system of this invention.

Example 2 - Synthesis or primers and the establishment of the detection system based on the 5' noncoding region and the core protein coding region.

(1) Synthesis of oligonucleotide primers.

Oligonucleotide primers (20 mer) were synthesized based on the 5' noncoding region sequences and the core protein coding region of strains HC-J1 and HC-J4 determined in Example 1. Oligonucleotide primer of HCV was also synthesized according to the nucleotide sequence disclosed in the European Patent Application No. 88310922.5 previously described. The model 3808 DNA Synthesizer (Applied Biosystems Japan) was used for such synthesis.

The number of primers synthesized were 20 (#3, 4, 5, 8, 9, 10, 11, 12, 16, 17, 21, 22, 23, 25, 32, 33, 34, 35, 36 and 48), and the position from the 5' terminus and nucleotide sequence for each of them is shown in Table 1.

(2) Isolation of NANB hepatitis viral RNA from a sample.

1 ml of a plasma sample was centrifuged on a model TL-100 (Beckman) ultracentrifuge at 9×10^4 rpm for

15 minutes and the precipitate thus obtained was suspended in buffer (containing 200 mM NaCl, 10 mM EDTA, 2% (w/v) sodium dodecyl sulfate (SDS) and proteinase K (1 mg/ml)) for incubation at 60°C for 1 hour.

Nucleic acids were extracted twice by using the same volume of phenol/chloroform and precipitated in ethanol at -20°C for over 3 hours. The precipitate was suspended in 70% ethanol for centrifugation and the precipitate was dissolved in 5 µl of distilled water after lyophilization.

(3) cDNA synthesis.

RNA extracted from a plasma sample in (2) above was denatured by heating at 70°C for 1 minute and cooled on ice before synthesis of cDNA. cDNA was synthesized by reverse transcription. 100 pmol each of antisense primers #5, 8, 11, 12, 16, 17, 25, 35, 36 and 48 were added with 4 kinds of deoxyribonucleoside 5'-triphosphates(Takara, Japan), 10 units of RNase Inhibitor (Takara, Japan) and 10 units of Reverse transcriptase AMV (Boehringer Mannheim, Germany), and incubated at 42°C for 90 minutes in a buffer (containing 10 µl of Tris chloride (50 mM, pH 8.4), 8 mM $MgCl_2$, 30 mM KCl and 1 mM dithiothreitol) to synthesize cDNA. cDNA thus obtained was purified by phenol/chloroform extraction.

(4) Amplification by PCR.

PCR was carried out using DNA Thermal Cycler (Perkin-Elmer Cetus) and DNA Amplification Reagent Kit (Perkin-Elmer Cetus) by the well-known method of Saiki et al. (1988). The reaction cycle of denaturalization (one minute at 94°C), annealing of primers (1.5 minutes at 55°C), and amplification of primers (3 minutes at 72°C) was repeated 35 times.

The PCR product was electrophoresed in a mixed agarose gel of 1-1.5% Nusieve and 1-1.5% Seakem (FMC), and, after staining with ethidium bromide, its bands were confirmed by ultraviolet radiation.

(5) Amplification by second-stage PCR.

The product obtained by the first pair of primers (#32 and #36, for example) by PCR can be subjected to second-stage PCR if necessary. As primers for such PCR, a pair of primers of nucleotides for regions within those of the first pair of primers (#33 and #48, for example) were chosen and PCR reaction cycle was repeated 30 times for 5 µl of the product obtained in the first-stage PCR. Reaction conditions for each cycle was denaturalization (1 minute at 94°C), annealing (1.5 minutes at 55°C), and amplification (2 minutes at 72°C). The product obtained in the second-stage PCR was electrophoresed and analyzed in the method described in (4) above.

Example 3 - Selection of pairs of primers effective for detection of NANB hepatitis virus by PCR.

Results of PCR test with two pairs of primers for 10 samples determined positive for HCV antibody (plasma samples nos. 1, 3, 5, 7 and 9 from Japanese blood donors and serum samples nos. 2, 4, 6, 8 and 10 from NANB hepatitis patients) are shown in Table 2.

PCR amplification was tried for 10 target nucleotide sequence regions; 2 regions each from NS5, NS3, and E-NS1 and its upstream (NS=nonstructural region, E-NS1=nucleotide region bridging the envelope region and nonstructural region number 1) referred to in Chiron's European Application), 2 regions each from the core region and 5' noncoding region identified under this invention.

As a result, when two pairs of primers from the 5' noncoding region (#32/#36 and #33/#48) and one pa
of primers from the core region (#23/#25) were used, expected sizes of NANB cDNA bands (242 bp, 145
and 377 bp) for respective regions were detected.

In the other 7 regions, however, only 2 to 9 out of 10 samples cold successfully be amplified, altho
presence of RNA itself was confirmed in each sample. It was therefore concluded that pairs of primer
and #33/#48 from the 5' noncoding region, and #23/#25 from the core region, were widely effective f
of HCV RNA. Thus, selection of primers from NS5, NS3 and E-NS1 regions coding for the nonstr
of the virus are quite insignificant.

In some cases, single-stage PCR is sufficient for detection of NANB hepatitis viral RNA.
ance sensitivity, two-stage PCR is recommended.

For example, samples which did not show the expected band of 242 bp when their c
using #38 primer and amplified by PCR with primers #36 and #32 (first-stage PCR) we
second-stage PCR using primers #33 and #48 and the first-stage PCR product as a
PCR). If the 145 bp band does not occur after second-stage PCR then the sample did no

Example 4

32 samples from chronic NANB hepatitis patients, 10 samples from chronic hepatitis B patients, and 12 samples from blood donors with normal ALT levels were tested for NANB hepatitis virus RNA by PCR. Results are shown in Table 3.

Preliminary test of 32 samples from NANB hepatitis patients showed 20 samples positive and 12 samples negative for anti-HCV. All 10 samples from hepatitis B patients and 12 samples from blood donors with normal ALT levels were negative for anti-HCV.

For the 20 samples out of the 32 total samples from NANB hepatitis patients which tested positive for anti-HCV, RNA was detected in 15 samples by the first-stage PCR and the remaining 5 by the second-stage PCR. Thus 100% of the samples (which tested positive for anti-HCV) tested positive by PCR.

Out of the 12 samples from NANB hepatitis patients which tested negative for anti-HCV, 7 samples by the first-stage PCR and 4 samples by the second-stage PCR (or 92%) turned out to be positive for NANB hepatitis viral RNA. All 10 hepatitis B cases and all 12 blood donor cases with normal ALT levels subjected to the test (so far as the second-stage PCR) were negative for the viral RNa. From these data, NANB hepatitis RNA detection system using oligonucleotide as primers has proven its excellent performance, and its two-stage PCR system in particular has proven its superb performance both in sensitivity (more than 50% higher than anti-HCV and detecting as much as 96.9% of NANB hepatitis viral RNA) and specificity.

(5) Sensitivity of the detection system for NANB hepatitis virus by cDNA/two-stage PCR.

Sensitivity of NANB hepatitis virus detection system by cDNA/two-stage PCR under this invention is described below. Results are shown in Table 4.

10-fold serially diluted samples of plasma (having known infectious unit of 10^7 CID/ml) were prepared and tested 3 times. In the first-stage PCR, the expected band of 242 bp was confirmed for 100 CID/ml in two tests and as low as 10 CID/ml in the remaining one test.

In the second-stage PCR, the expected band of 145 bp was confirmed for 10 CID/ml in two tests and as low as 1 CID/ml in one test. No band was detected for concentrations less than 1 CID/ml or for negative samples.

Average titer of NANB hepatitis patients is estimated to be 10^{2-4} and the described detection system is considered to give clinically significant sensitivity for diagnosis of NANB hepatitis patients.

The present invention thus provides a highly sensitive and specific detection system for NANB hepatitis virus. Accordingly, this invention will become instrumental in accurate diagnosis of hepatitis patients and screening of donor blood for prevention of posttransfusion hepatitis.

The present invention also concerns a nucleotide sequence of NANB which contains at least a portion of the bases 1-1863 described above. The sequence is constituted of a plurality of nucleotides and contains at least one primer as shown in table 1. In addition, the sequence is terminated at least at one end with a primer as shown in table 1.

The present invention further concerns a method of detecting non-A, non-B hepatitis virus comprising:

(1) synthesizing cDNA from viral RNA;

(2) amplifying said cDNA by PCR in first stage to produce a product;

(3) amplifying the product by PCR in a second stage.

The amplifying is carried out by using at least one oligonucleotide primer according to claim 8. The primer in step (1) may be a pair of primers and the primer in step (2) may be a pair of primers from a region within the pair of primers in step (1). The pair of primers in step (1) may be primers #32 and #48 in table I and the pair of primers in step (2) may be primers #33 and #48 in table 1. The pairs of primers #32/#36, #33/48, and #23/#25 in table 1.

The present invention also concerns diagnostic test kits for detection NANB in biological samples, including for example blood and serum samples. The test kit includes (1) at least one primer derived from the nucleotide sequence disclosed above, (2) dATP, dTTP, dGTP, and dCTP; and (3) heat stable DNA polymerase. Kits suitable for diagnosis and NANB and containing the appropriate reagents are constructed by packaging the appropriate materials, including the primer in suitable containers, along with the remaining reagents and materials required, as well as a suitable set of instructions for conducting the test.

Further variations and modifications of the invention will become apparent to those skilled in the art from the foregoing and are intended to be encompassed by the claims appended hereto.

Japanese Patent Application No. Heisei 2 Nen 153402, filed on June 12, 1990, is relied on and incorporated by reference.

Primers	Nucleotide Position	Nucleotide Sequences
#3	126−145	AAACCTTGCGGTATTGTGCC
#4	153−172	AGTGTGTGTGGTCCGGTATA
#5	268−287	CGGTGCCCTGGTATTGTTAA
#6	303−322	GAGTTCATCCAGGTACAACC
#9	6427−6446	AGATGGCTTTGTACGACGTG
#10	6490−6509	TCCAATACTCACCAGGACAG
#11	6761−6780	CACAGCTAGTTGTCAGTACG
#12	6786−6805	TTGATGTAGCAAGTGAGGGT
#16	4029−4048	CTGGTGACAGCAGCTGTAAA
#17	4061−4080	TGAAGACGAGGGTTTGGCTA
#21	3669−3688	TATTGCCTGTCAACAGGCTG
#22	3759−3778	CGAGAGTTCGATGAGATGGA
#23	450−469	TAGATTGGGTGTGCGCGCGA
#25	807−826	TCCCTGTTGCATAGTTCACG
#32	7−26	ACTCCACCATAGATCACTCC
#33	46−65	TTCACGCAGAAAGCGTCTAG
#34	475−494	AAGACTTCCGACCGGTCGCA
#35	568−587	TTGCCATAGAGGGGCCAAGG
#36	229−248	AACACTACTCGGCTAGCAGT
#48	171−190	GTTGATCCAAGAAAGGACCC

Table 2: Detection of NANB hepatitis viral RNA in HCV antibody positive samples by PCR using various sets of primers.

Primers / Sample No.	5' noncoding Region		presumable core gene		E−NS1		NS3		NS5	
	#32 / #36 (242bp)	#33 / #48 (145bp)	#23 / #25 (377bp)	#34 / #35 (113bp)	#3 / #6 (197bp)	#4 / #5 (135bp)	#21 / #17 (412bp)	#22 / #16 (290bp)	#9 / #12 (379bp)	#10 / #11 (291bp)
1	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	−	+	+	+	+	−
3	+	+	+	+	−	+	+	+	+	−
4	+	+	+	+	−	+	−	+	+	−
5	+	+	+	+	−	−	−	+	+	−
6	+	+	+	+	−	+	+	+	+	−
7	+	+	+	+	−	−	−	+	+	−
8	+	+	+	+	−	−	−	+	+	−
9	+	+	+	−	−	+	−	−	−	−
10	+	+	+	−	−	+	−	−	−	−

Samples Nos. 1, 3, 5, 7 and 9 are taken from blood donors, and samples Nos. 2, 4, 6, 8 and 10 are taken from chronic NANB hepatitis patients.

Table 3: NANB hepatitis viral RNA detection by cDNA/two-stage PCR. One pair each of primers #32 and 38, and #33 and #48 was used for the first-stage and second-stage PCR respectively.

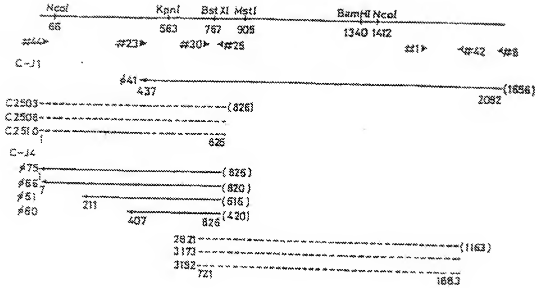
Source of Samples	Total number of Samples	Number of samples positive for anti-HCV by ORTHO's EIA	Number of samples positive for NANB hepatitis viral RNA		
			1st-stage PCR	2nd-stage PCR	Total (%)
Chronic NANB hepatitis	32	20 (62.5%)	22	9	31 (96.9%)
Chronic hepatitis B	10	0 (0)	0	0	0 (0)
Blood donors with normal ALT level	12	0 (0)	0	0	0 (0)

Table 4: Detection of NANB hepatitis viral RNA by two-stage PCR in samples with known infectivity titers. One pair each of primers #32 and #36, and #33 and #48 were used for the first-stage and second-stage respectively.

Test	PCR	*Serial Dilution (CID/ml)							Control (Negative)
		$\times 10^2$ (10^5)	$\times 10^3$ (10^4)	$\times 10^4$ (10^3)	$\times 10^5$ (10^2)	$\times 10^6$ (10)	$\times 10^7$ 1	$\times 10^8$ 0.1	
First Test	1st-stage	+++	+++	++	+	-	-	-	-
	2nd stage	NT	NT	NT	+	+	-	-	-
Second Test	1st-stage	+++	+++	++	+	+/-	-	-	-
	2nd-stage	NT	NT	NT	+	+	+	-	-
Third Test	1st-stage	+++	+++	++	+	-	-	-	-
	2nd-stage	NT	NT	NT	+	+	-	-	-

Same plasma negative for HCV antibody and HCV RNA was used as diluent and control. CID means Chimpanzee Infectious Dose.

Fig. 1



Claims

1. A nucleotide sequence of non-A, non-B hepatitis virus comprising [bases 1-1863].

2. The nucleotide sequence of non-A, non-B hepatitis virus according to claim 1, comprising [bases 1-1672].

3. The nucleotide sequence of non-A, non-B hepatitis virus according to claim 1, comprising [bases 1-324].

4. The nucleotide sequence of non-A, non-B hepatitis virus according to claim 3, wherein the 187th nucleotide A is replaced by C, the 217th nucleotide C is replaced by T, and the 226th nucleotide A is replaced by G.

5. The nucleotide sequence of non-A, non-B hepatitis virus according to claim 1, comprising [bases 325-1863].

6. The nucleotide sequence of non-A, non-B hepatitis virus according to claim 1, comprising [bases 325-864].

7. The nucleotide sequence of non-A, non-B hepatitus virus according to claim 1, comprising [bases 865-1476].

8. An oligonucleotide primer derived from the nucleotide sequence of non-A, non-B hepatitis virus according to claim 1.

9. The oligonucleotide primer according to claim 8, wherein said primer contains from 15 to 25 nucleotides.

10. The oligonucleotide primer according to claim 9, wherein said primer contains 20 nucleotides.

11. The oligonucleotide primer according to claim 8, wherein said primer comprises primer #23 in table 1.

12. The oligonucleotide primer according to claim 8, wherein said primer comprises primer #25 in table 1.

13. The oligonucleotide primer according to claim 8, wherein said primer comprises primer #32 in table 1.

14. The oligonucleotide primer according to claim 8, wherein said primer comprises primer #33 in table 1.

15. The oligonucleotide primer according to claim 8, wherein said primer comprises primer #36 in table 1.

16. The oligonucleotide primer according to claim 8, wherein said primer comprises primer #48 in table 1.

17. An oligonucleotide primer derived from the nucleotide sequence of non-A, non-B hepatitis virus according to claim 3.

18. An oligonucleotide primer derived from the nucleotide sequence of non-A, non-B hepatitis virus according to claim 7.

19. A method of detecting non-A, non-B hepatitis virus comprising synthesizing of cDNA from viral RNA and amplifying said cDNA by PCR using at least one oligonucleotide primer according to claim 8.

20. The method according to claim 19, wherein said oligonucleotide primer is selected from the group consisting of: primer #23 in table 1, Primer #25 in table 1, primer #32 in table 1, primer #33 in table 1, primer #36 in table 1, and primer #48 in table 1.

21. The method according to claim 20, wherein said oligonucleotide primers consists of primer #32 in table 1 and primer #36 in table 1.

22. The method according to claim 20, wherein said oligonucleotide primers consists of primer #33 in table 1 and primer #48 in table 1.

23. The method according to claim 20, wherein said oligonucleotide primers consists of primer #23 in table 1 and primer #25 in table 1.

24. An oligonucleotide primer derived from the nucleotide sequence of non-A, non-B hepatitis virus according to claim 6.

25. A test kit for diagnosing non-A, non-B hepatitis or for detecting non-A, non-B hepatitis virus, said kit comprising:
1) at least one primer according to claim 8;
2) dATP, dTTP, dGTP, and dCTP; and
3) heat stable DNA polymerase.

26. A nucleotide sequence of non-A, non-B hepatitis virus comprising at least a portion of the bases 1-1863, said sequence being constituted of a plurality of nucleotides and containing at least one primer as shown in table 1.

27. A nucleotide sequence of non-A, non-B hepatitis virus comprising at least a portion of the bases 1-1863, said sequence being constituted of a plurality of nucleotides and terminated at least at one end with a primer as shown in table 1.

28. A method of detecting non-A, non-B hepatitis virus comprising:
(a) synthesizing cDNA from viral RNA;
(2) amplifying said cDNA by PCR in a first stage to produce a product;
(3) amplifying said product by PCR in a second stage; said amplifying being carried out by using at least one oligonucleotide primer according to claim 8.

29. The method according to claim 28, wherein said primer in step (1) is a pair of primers and said primer in step (2) is a pair of primers, wherein said pair of primers in step (2) are from a region within the pair of primers in step (1).

30. The method according to claim 29, wherein said pair of primers in step (1) are primers #32 and #48 in table 1 and said pair of primers in step (2) are primers #33 and #48 in table 1.

31. The method according to claim 28, wherein said pairs of primers are selected from pairs of primers #32/#36, #33/#48, and #23/#25 in table 1.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 91305270.0

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
P,X	EP - A1 - 0 388 232 (CHIRON CORPORATION) * Abstract; claims 15-18 *	1,3,6, 7,25, 26,28	C 07 H 21/04 C 12 Q 1/70 C 12 Q 1/68 C 12 N 15/51 C 12 P 19/34
D,A	EP - A1 - 0 318 216 (CHIRON CORPORATION) * Claims 1-5,21,29 *	26,28	
P,A	EP - A2 - 0 398 748 (CHIRON CORPORATION) * Abstract; claims 1-4,7,15, 16,18 *	1,3,8, 19,25, 28	
A	WO - A1 - 90/00 597 (GENELABS INCORPORATED) * Abstract; claims 10-13,27 *	1,19, 28	
A	CHEMICAL ABSTRACTS, vol. 110, no. 25, June 19, 1989, Columbus, Ohio, USA QU. L. CHOO et al. "Isolation of cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome" page 145; column 1, abstract-no. 226 247n & Science (Washington D.C., 1881-) 1989, 244(4902), 359-62	1	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C 07 H C 12 Q C 12 N C 12 P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	12-09-1991	SCHNASS

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document